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Characterization of a Phenylglyoxal-sensitive Passive Ca^{2+} Permeability in Human Erythrocytes

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Abstract. In viable human erythrocytes, passive downhill entry of Ca^{2+} through the plasma membrane is opposed by an active extrusion Ca^{2+}-pump mechanism which maintains a free $[Ca^{2+}]_i \approx 10^{-7}$ M. This passive Ca^{2+} permeability can be determined reliably in cells with a completely arrested Ca^{2+} pump, as can be achieved in cells which are both ATP-depleted and vanadate-treated. With this approach, whole-cell preparations from banked human erythrocytes exhibited passive net uptake of Ca^{2+} that has characteristics of a carrier-mediated process which could be shunted by the addition of the Ca^{2+} ionophore A23187. Passive Ca^{2+} uptake was apparently independent of extracellular Na^+, K^+ or Cl^- and was not responsive to a variety of Ca^{2+} channel entry blockers. However, in the presence of phenylglyoxal, a specific, covalent modifier of arginyl residues, the initial (0 to 60 min) rate of passive Ca^{2+} uptake was inhibited in a dose-dependent manner. This can be taken in support of a proteinaceous carrier-mediated passive Ca^{2+} entry mechanism that involves one or several functional arginyl residue(s) and may represent Ca^{2+} back-diffusion through the arrested Ca^{2+} translocation pump.

Key words: Passive Ca^{2+} permeability — Human erythrocyte membrane — Phenylglyoxal — strontium — Ca^{2+} channel entry blockers

Introduction

Human and other non-nucleated erythrocytes maintain a free intracellular Ca^{2+} concentration of about 10^{-7} M or less in a resting, i.e., noncirculating state. This concentration may be temporarily increased in erythrocytes that are physically stressed, either in an *in vivo* microcirculatory situation or by artificially induced shear stress *in vitro* (Larsen et al., 1981).

Three basic mechanisms are responsible for the maintenance of this relatively low free intracellular Ca^{2+} concentration. They are (i) an inherently low passive permeability for extracellular Ca^{2+}, (ii) various intracellular Ca^{2+}-binding components such as calmodulin and polar phospholipid heads on the inner leaflet of the plasma membrane and (iii) a primary active Ca^{2+}-transport extrusion mechanism (Ca^{2+} + Mg^{2+})-ATPase activity. Under normal resting conditions this Ca^{2+}-transport system operates at approximately 1% of its maximal capacity, suggesting that under normal operating conditions for an erythrocyte in circulation, the system is well designed to extrude much larger quantities of Ca^{2+} stemming from a compromised passive permeability barrier. This passive permeability barrier may be compromised by acquired or inherent pathological conditions, as well as by the presence of certain xenobiotics and drugs.

In the past, measuring passive Ca^{2+} permeability has been attempted using intracellular Ca^{2+} chelator loading, or various vanadate pretreatment protocols or metabolic inhibition by substrate depletion. Desai, Schlesinger and Krogstad (1991) used a combination of metabolic ATP depletion (with iodoacetamide) and vanadate treatment to obtain a physiologic rate of Ca^{2+} entry, in the absence of any residual Ca^{2+} extrusion pump (Ca^{2+} + Mg^{2+})-ATPase activity. Furthermore, these authors suggested that net passive Ca^{2+} entry was mediated via a simple carrier mechanism obeying Michaelis-Menten kinetics at concentrations below 5 mM Ca^{2+} with a V_{max} of 291 to 321 nmol Ca/g dry weight per hr and a K_m of 1.1 to 1.3 mM Ca^{2+}. An additional, unsaturable component of less than 5% of V_{max} between 5 and 30 mM Ca^{2+} was also observed (Desai et al., 1991). Similar two-component influx kinetics using different experimental conditions have been observed by other laboratories as well (Tiffert,

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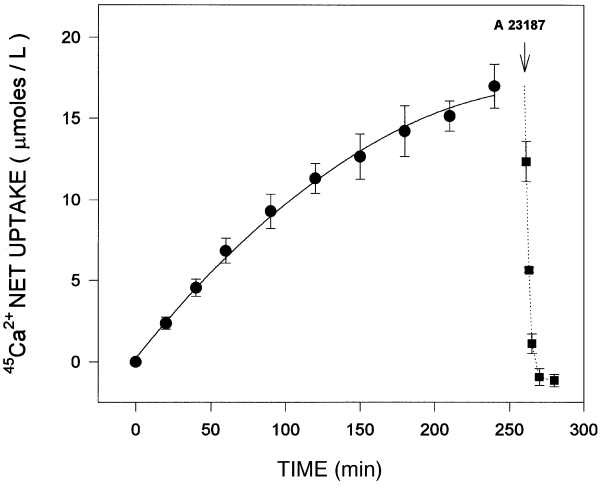

Fig. 1. Passive Ca^{2+} permeability and the effect of A23187 in human erythrocytes: Net uptake of $^{45}Ca^{2+}$ into metabolically depleted and vanadate-pretreated erythrocytes. After 4 hr of accumulation and removal of extracellular Ca^{2+}, 1 μM A23187 ionophore was added to the incubation medium. Data shown are the means and SD of two independent experiments carried out in duplicate. The curve shown is a least square regression fit of the 2nd order with a correlation coefficient (*r*) of 0.986. Where missing error bars were smaller than symbol size.

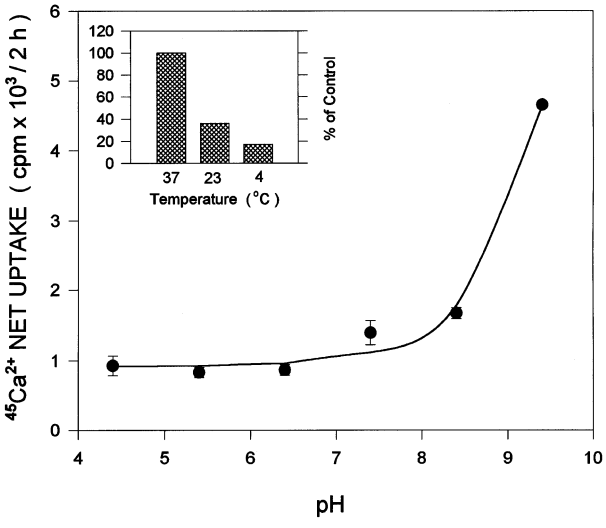

Fig. 2. Effects of temperature and extracellular pH on passive Ca^{2+} permeability into erythrocytes: Incubation temperature and hydrogen ion concentrations were changed for 180 min and the rate of $^{45}Ca^{2+}$ net uptake was assessed by regression analysis and expressed as % of control at 37° and pH 7.4 (inset). The pH profile was assessed by measuring total accumulation of ^{45}Ca after 2 hr at 37° and is expressed as the means and SEM of accumulated counts in 3 independent experiments.

Garciá-Sancho & Lew, 1984; McNamara & Wiley, 1986; Lew, 1990).

In the present work, we examined passive Ca^{2+} permeability in human erythrocytes under conditions that maximized the probability of a completely arrested extrusion mechanism. In addition, we characterized the carrier in terms of temperature, ion dependency, Sr^{2+} competition, Ca^{2+} channel entry blocker sensitivity and irreversible pharmacological modification by phenylglyoxal.

Materials and Methods

Preparation of Substrate-depleted Erythrocytes

Outdated human erythrocytes (1 to 35 days past the expiration date) from the local blood bank were washed and centrifuged 3 times in about four volumes of ice-cold 154 mM NaCl at 5,000 rpm. After each centrifugation the supernate and top layer of contaminating lipids, white cells and platelet debris were carefully removed to give a homogeneous sample of red blood cells. The hematocrit was determined (typically >94%) and adjusted to 5% with a buffer (pH 7.4) containing (in mM): 130 KCl, 30 sucrose, 20 Hepes, 6 iodoacetamide, 10 inosine and 0.1 EGTA. This cell suspension was incubated for three hours at 37°, at which point cells could be stored on ice for up to 3 days without any noticeable change in passive permeability characteristics.

Measurement of Passive $^{45}Ca^{2+}$ Uptake

Cells suspended in depletion buffer were incubated at 37° for 30 min in the presence of 0.5 mM Na_3VO_4. Passive uptake was then initiated by the addition of 2 mM $^{45}CaCl_2$ (specific activity 1.2 to 2.4 μc/mg) to a 4% cell suspension in depletion buffer containing 0.5 mM sodium vanadate and various additions. Modifications and additions to this standard protocol are noted in the appropriate result section and figure legends. At various timed intervals a 200 μL sample was removed and quenched in six volumes ice-cold buffer containing 1.0 mM EGTA. The samples were then centrifuged for 9 sec in an Eppendorf microfuge. The pellet was washed twice more in this solution and then bleached and digested with 200 μL of 2.5% NaOCl solution. Zero time samples served as background measurements, and cell numbers determined by Coulter Counter or with a hemocytometer were used to calculate uptake rates and total cell volume based on an average cell volume of 80 fl.

Unless otherwise stated, graphs show computer-fitted least square regression lines of the second order, and error bars in graphs represent SEM of at least three independent experiments (or SD in cases involving only two experiments) carried out in duplicate or triplicate. If missing, values for error bars are smaller than symbol size. Significance of differences were evaluated using Student's *t*-test analysis.

Results

Net uptake of Ca^{2+} into Metabolically Arrested, Ca^{2+} Pump-inhibited Erythrocytes

Erythrocytes that were pretreated under conditions that insure a completely inactivated Ca^{2+} extrusion pump, i.e., ATP-depleted and sodium vanadate-pretreated (Desai et al., 1991), were incubated for an additional 4 hr in standard iso-osmotic (302 mOsm) incubation medium containing (in mM): 2 $^{45}CaCl_2$, 130 KCl, 30 sucrose, 20

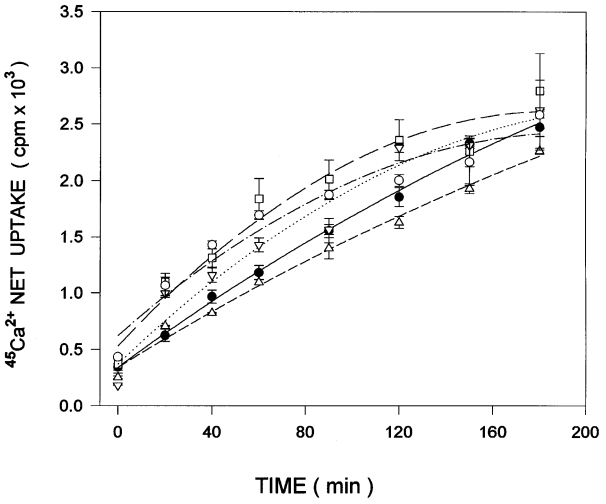

Fig. 3. Nondependence on potassium or sodium ion: Rates of erythrocyte Ca^{2+} net uptake in media with various ionic compositions. (in mM): 130 KCl (control, ●); 130 NaCl (□); 126 NaCl, 4 KCl (○); 126 KCl, 4 NaCl (△); and 130 choline chloride (▽). Curves are least square fitted 2nd order regressions through means of 2 to 5 (control) experiments.

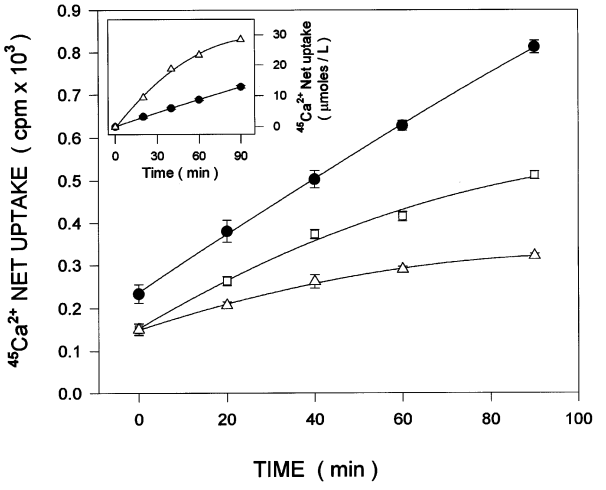

Fig. 4. Ca^{2+} net uptake competition by strontium: Time course of Ca^{2+} net uptake in the absence and presence of an additional 15 mM nonradionuclide $CaCl_2$ (△) or $SrCl_2$ (□) to control (2 mM $^{45}CaCl_2$) incubation medium (●). The inset graph shows absolute uptake corrected for specific radioactivity in the presence of 2mM (●) and 15 mM $CaCl_2$ (△).

Hepes, 6 iodoacetamide, 10 inosine, 0.1 EGTA and 0.5 Na_3VO_4. Figure 1 shows a slow, gradually curvilinear influx of Ca^{2+} into the cells with a rate of influx of 6.85 ± 0.38 μM/L cells/hr during the initial 60-min period. After 4 hr of intracellular Ca^{2+} accumulation, extracellular Ca^{2+} was removed by washing the cells in incubation medium lacking free Ca^{2+}. These cells could then be shunted efficiently and rapidly by the addition of 1 μM calcium ionophore A23187 to the medium ($t_{1/2}$ 2.48 min from a fitted first order exponential curve). After 10 min, intracellular levels decreased to pre-accumulation levels.

Temperature and pH Dependence of Passive Ca^{2+} Permeability in Erythrocytes

Lowering the normal incubation buffer temperature of 37° to 23° and 4° diminished the rate of passive uptake over a 3-hr period to 36% and 17%, respectively (inset Fig. 2).

Altering the pH in the incubation buffer from 4.4 to 6.4 did not affect the uptake rate significantly. Raising the pH further, however, produced an apparently exponential increase resulting in a 5.5-fold greater rate of Ca^{2+} uptake at pH 9.4.

Sodium, Potassium and Chloride Independence of Ca^{2+} Net Uptake

Figure 3 summarizes passive erythrocyte Ca^{2+} net uptake rates under various depletion and incubation conditions with regard to ion composition. To explore the cation dependence of the uptake process, the standard 130mM KCl was substituted with 130 mM NaCl, or 130 mM choline chloride, or the combinations of 126 mM KCl and 4 mM NaCl, or 126 mM NaCl and 4 mM KCl. The time course over 180 min was curvilinear as seen in Figs. 1 and 3, and under conditions with the higher amounts of sodium (□, ○) the curvature was somewhat more pronounced, suggesting that the uptake process would reach a plateau earlier than under conditions that lacked any sodium. Similar rates of uptake were obtained when chloride ions were substituted with sulfate in the form of K_2SO_4 in concentrations ranging from 35 to 104 mM with K^+ kept constant (*not shown*). Osmolarities of the various incubation media were calculated and then measured (288 to 308 mosm/L) to minimize contributions of changes in cell volume. Thus, rate of Ca^{2+} net uptake were largely unaffected by changing extracellular main ion composition ruling out several exchange and channel pathways.

Strontium Competition With Ca^{2+} for Entry into the Cell

Figure 4 demonstrates the effects of the addition of 15 mM nonradionuclides $SrCl_2$ or $CaCl_2$ to the incubation medium containing 2 mM $^{45}CaCl_2$. The apparent inhibition of net uptake in terms of cpm by $CaCl_2$ is on account of the dilution of specific activity, whereas $SrCl_2$ seemingly competed with $CaCl_2$ for entry into cells and reduced it by 55% after 90 min. The comparatively lesser inhibition by a equimolar concentration of $SrCl_2$ suggests that this particular ion has a decreased affinity for the permeability site. The inset in Fig. 4 shows the actual

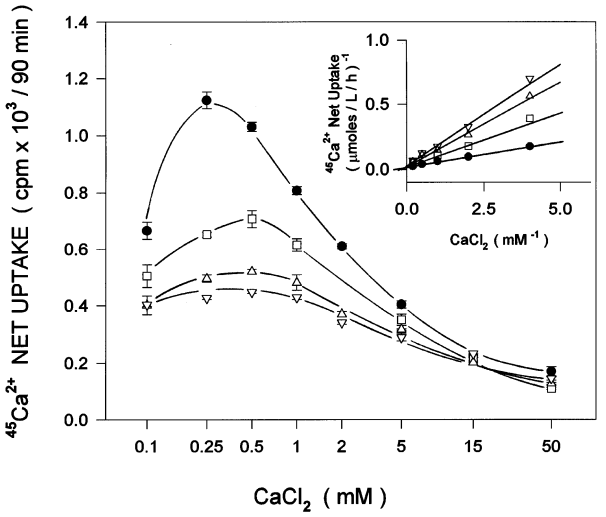

Fig. 5. Concentration-dependent Sr^{2+} competition for Ca^{2+} net uptake: Semi-logarithmic Ca^{2+} concentration-effect relationship curves in the absence (●) and the presence of 5 mM (□), 15 mM (△) and 30 mM (▽) strontium chloride. Accumulation of $^{45}Ca^{2+}$ over a 90-min period is shown with the inset representing a double-reciprocal plot of net Ca^{2+} uptake rates in the range of 0.1 mM to 5 mM substrate after correction for specific activity.

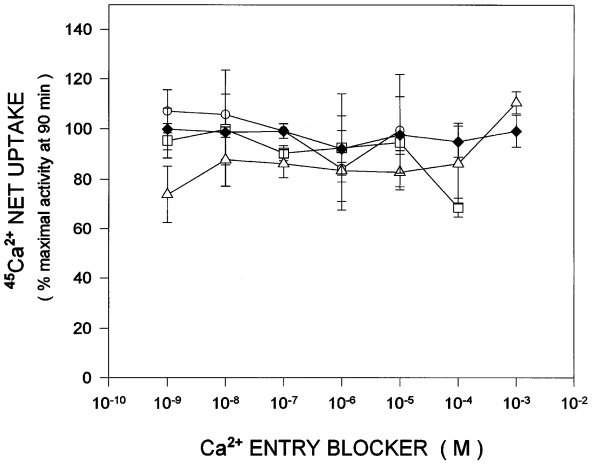

Fig. 6. Lack of inhibition of Ca^{2+} channel entry blockers: $^{45}Ca^{2+}$ net uptake into ATP-depleted, vanadate-treated erythrocyte is unaffected by a wide concentration range of verapamil (●), diltiazem (△), nifedipine (○) and bepridil (□). Data are expressed as mean percents and SD of maximal activity at 90 min in the absence of any Ca^{2+} antagonists from single experiments performed in duplicate.

increased rate of Ca^{2+} uptake (corrected for specific activity) due to the addition of 15 mM $CaCl_2$. Predictably, the time to equilibrium of passive influx over passive efflux was diminished by increasing the ratio Ca_{ic}/Ca_{ec}.

Accumulation of counts at 90 min as a function of increasing Ca^{2+} concentrations is shown in Fig. 5. At all Ca^{2+} concentrations up to 5 mM, strontium inhibited the uptake process in an apparently competitive manner (K_{Ca} = 1.3 mM and K_i for Sr^{2+} = 7.8 mM).

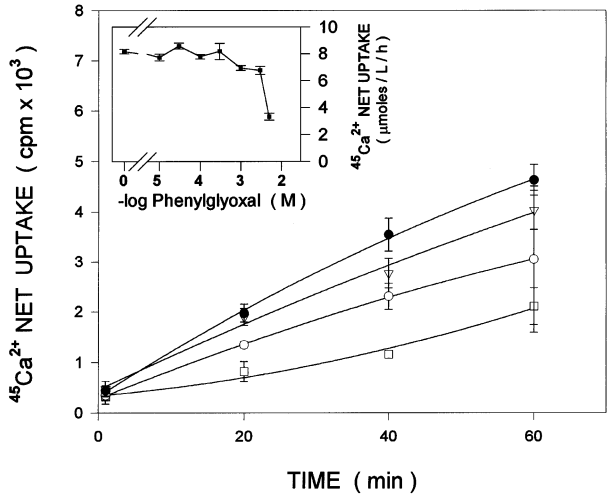

Fig. 7. Effects of phenylglyoxal on passive Ca^{2+} permeability: Inhibition of the initial rate of $^{45}Ca^{2+}$ net uptake (●) by 1 mM (▽), 3 mM (○) and 10 mM (□) phenylglyoxal. The curves shown are computer-fitted 2nd order polynomial regressions. Inset: Concentration-effect relationship of a 90-min exposure to phenylglyoxal expressed as µmoles/L cells/hr.

Lack of Inhibition by Ca^{2+} Channel Entry Blockers

Several prototype Ca^{2+} channel entry blockers with a large diversity in their physicochemical characteristics failed to significantly reduce (P 0.05) the passive Ca^{2+} permeability over a wide range of concentrations (Fig. 6). Similarly, ryanodine (10^{-12} to 10^{-6} M), the conotoxins GVIA and MVIIC (10^{-12} M to 10^{-6} M), and the potassium channel modulators TEA and TMA (10^{-7} M to 10^{-3} M) did not affect the rate of passive Ca^{2+} entry (*not shown*).

Inhibition of Passive Ca^{2+} Net Uptake by Phenylglyoxal

Phenylglyoxal, inhibited passive Ca^{2+} permeability in a concentration-dependent manner. Figure 7 shows a 60-min time course in the presence of 1, 3, and 10 mM phenylglyoxal with the concentration-effect relationship shown in the inset. After a 90-min exposure to 5 mM phenylglyoxal net passive $^{45}Ca^{2+}$ permeability was reduced by 60% from 8.17 ± 0.17 to 3.32 ± 0.26 µmoles/L cells/hr.

Discussion

It is generally accepted that mammalian cells need to very tightly control a typically low intracellular level of

calcium. The primary active Ca^{2+} extrusion pump in the plasma membrane is an important component in this homeostasis (Campbell, 1983; Schatzmann, 1983; Rega, 1986; Stein, 1986; Lew, 1990). Whereas the behavior of this pump mechanism is relatively well understood, the nature and characterization of passive fluxes has been only slowly forthcoming. However, the use of ATP-depleted, iodoacetamide- and vanadate-pretreated cells which insures complete inhibition of the Ca^{2+} extrusion pump, allowed for the first time to convincingly assess and characterize what appears to be a carrier-mediated, reversible transport process that can also transport strontium (Desai et al., 1991). Using this approach, we examined the passive Ca^{2+} permeability in human erythrocytes under a variety of physiological and pharmacological conditions to further characterize the process and to confirm or rule out previous suggestions that these membranes contain a dihydropyridine or verapamil inhibitable, L-type Ca^{2+} channel (Varecka & Carafoli, 1982; Neyses et al., 1985; Morris, David-Dufilho & Devynck, 1988; Engelmann & Duhm, 1989).

The rate of Ca^{2+} entry into the simultaneously ATP-depleted and vanadate-pretreated outdated cells is on the order of 7 to 9 μM/L cells/hr (at Ca^{2+}_{ec} = 2.0 mM) which is in reasonably good agreement with previous estimates using a variety of methodologies (Ferreira & Lew, 1977; Varecka & Carafoli, 1982; McNamara & Wiley, 1986) for measuring passive entry but considerably lower than the 53 μM/L/hr found by Desai et al. (1991) employing the method used here. These authors claimed that the lower rates of Ca^{2+} entry found by others were due to an incompletely arrested active extrusion pump and thus resulting in an imprecise saturability level, i.e., a nonsaturating leak offset by residual Ca^{2+} transport activity. Similarly, Lew et al. (1982) found rates of Ca^{2+} entry into chelator loaded cells of 48 μM/L/hr when using autologous plasma as an incubation medium but only 17 μM/L/hr when using a saline-based incubation medium. Our own work measuring passive Ca^{2+} entry in freshly prepared erythrocyte vesicles of normo- and hyperlipidemic rabbits would tend to corroborate this work and strongly suggested an important role for plasma lipids in the regulation of plasma membrane Ca^{2+} transport, both passive and active (Raess, Porro & Tunnicliff, 1995). However, the simple absence or the presence of plasma lipoproteins, and the fact that we employ banked, 0–5-week outdated packed red cells in this study, cannot explain the difference in rates reported here and those obtained by Desai et al. (1991). Side-by-side comparison of freshly drawn *vs.* banked blood resulted in similar rates of net Ca^{2+} uptake. In fact, fresh cells in a single experiment, showed a net accumulation of only 79% and 82% of that of outdated cells after 60 and 240 minutes, respectively (*not shown*).

At 2 mM Ca^{2+}_{ec} net passive Ca^{2+} uptake fitted to an exponential function reaches 97% of the plateau of 21.36 μmoles/L cells at 600 min and can be short-circuited efficaciously (with a $t_{1/2}$ of 2.5 min) by the addition of 1 μM Ca^{2+} ionophore A23187 (calcimycin), suggesting that Ca^{2+} accumulates into a mobile and releasable pool of free intracellular Ca^{2+}.

The net uptake could be shown to be mostly unaffected by changing the pH within one unit of normalcy (7.4) but was clearly temperature sensitive as expected from a phospholipid-embedded carrier-mediated process that does not participate in proton exchange. On the basis of substituting sodium and potassium completely, and in various ratios, without affecting rates of uptake significantly, a Na^+/Ca^{2+} exchange mechanism was ruled out, as was a chloride dependency by sulfate ion substitution. However, as shown in Figs. 4 and 5 and by Desai et al. (1991) strontium chloride can compete for Ca^{2+} entry but appears to be a less preferred substrate. Similarly, but to a considerably lesser extent, 15 mM $MgCl_2$ was capable of inhibiting Ca^{2+} net uptake below 2 mM external $CaCl_2$ (*not shown*) resulting in an apparent $Ca^{2+} > Sr^{2+} > Mg^{2+}$ selectivity for the uptake process.

Several reports, using different approaches to study passive Ca^{2+} influx, have suggested that there may be Ca^{2+} entry blocker-sensitive Ca^{2+} channels in the human erythrocyte membrane (Varecka & Carafoli, 1982; Neyses et al., 1985; Morris, David-Dufilho & Devynck, 1988; Engelmann & Duhm, 1989). In addition to differences in cell sources and experimental protocols that according to others (Engelmann & Duhm, 1989; Desai et al., 1991) did not insure a completely arrested extrusion mechanism, many of the previously reported observations attributed to calcium channel inhibition can be simply explained by the modulation of residual Ca^{2+} pump activities or nonspecific, membrane-perturbing effects of the entry blockers or possibly their solvent vehicles (Raess & Record, 1990). Alternatively, the source and storage conditions of the cells used in this and other studies needed exploring. It appears that tissue and membrane composition, and levels of cholesterol and other lipids, are related to the plasma lipoprotein profile, and that this composition is important in the maintenance of integrity of membrane permeability barriers in general (*for review see* Yeagle 1988; Luly 1989) and for Ca^{2+} in particular (Vincenzi, 1989; Richter, 1992). In addition to data shown in Fig. 6, and to specifically rule out differences in sensitivity of the passive permeability to Ca^{2+} channel entry blockers on account of differences between freshly drawn and outdated red cells, we compared outdated, banked cells with freshly drawn cells from a single donor in their response to the effects of verapamil (up to 10^{-3} M). We found verapamil to be without any inhibitory effects in either preparation. This suggests that the lack of sensitivity to Ca^{2+} channel entry blockers is not a question of channel liability. Furthermore, to

rule out the possibility of Ca^{2+} entry through other cation channels typically found in excitable tissues, we tested ryanodine, conotoxins and potassium channel modulators as well. None of the compounds tested, over large concentration ranges, affected the Ca^{2+} net uptake described here.

It has been shown by Wüthrich, Schatzmann & Romero (1979) that under contrived experimental conditions the primary active extrusion pump can be reversed, i.e., a large Ca^{2+} transport gradient in the opposite direction can be used to generate ATP. Given the small but finite, presumably carrier-mediated, Ca^{2+} net uptake in cells with a disabled active Ca^{2+} transport pump, and in the absence of any functional Ca^{2+} entry channels (as defined by a lack of sensitivity to various pharmacological agents), no additional channel or exchange mechanisms need to be postulated to explain the Ca^{2+} permeability. With this precedence of a reversible ion-translocation mechanism, it is not inconceivable that the Ca^{2+} entry observed in our studies is taking place using the arrested pump as a carrier. This notion is supported by our observations that phenylglyoxal can inhibit the process in a dose-dependent manner. Phenylglyoxal, an irreversible carbodiimide arginyl modification agent, has been shown convincingly to inhibit a number of membrane transport processes including (Ca^{2+} + Mg^{2+})-ATPase activity and the Ca^{2+} transport pump (Raess, Tunnicliff & Record, 1985; Raess, 1993). From this work, we suspect that there is a functionally essential arginyl group in the active site of the ATPase which is protected by the substrate ATP and thus prevents phenylglyoxal from covalently modifying the enzyme. Moreover, based on the kinetic analysis of the inhibition of the enzyme by phenylglyoxal, the possibility exists that the regulatory ATP binding site is modified as well. Since there is no ATP present in the cellular preparations used in this study, it is likely that these same sites are modified covalently since the estimated K_i of the passive flux is in good agreement with our previous studies of the primary active (Ca^{2+} + Mg^{2+})-ATPase activity and Ca^{2+} transport (Raess et al., 1985; Raess, 1993). Unfortunately, an ATP ''protection protocol'' in this experimental setup is not possible, because the addition of ATP to these depleted cells negates any Ca^{2+} net uptake on account of reactivating the extrusion pump.

Notwithstanding the absence of a direct measurement of phenylglyoxal incorporation into the translocation enzyme, we contemplate the idea that the passive Ca^{2+} permeability studied here may well be a back-diffusion of the ion through its idle extrusion pump. This suggestion is consistent with both the present findings and other proposals of a saturable carrier-mediated Ca^{2+} net entry without exchange or ion codependence (Ferreira & Lew, 1977; Lew et al., 1982; Desai et al., 1991). Alternatively, another as of yet unidentified proteinaceous carrier with characteristics described here cannot be ruled out unequivocally. Therefore, further work will be needed to establish the correlation of phenylglyoxal incorporation into the pump and inhibition of this passive permeability of erythrocytes to Ca^{2+}.

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